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Targeted Mutagenesis of the Endogenous Mouse *Mis* Gene Promoter: In Vivo Definition of Genetic Pathways of Vertebrate Sexual Development

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Summary

Mutations were introduced into conserved steroidogenic factor 1 (SF1)– and SOX9-binding sites within the endogenous mouse *Müllerian inhibiting substance* (*Mis*) promoter. Male mice homozygous for the mutant SF1-binding site correctly initiated *Mis* transcription in fetal testes, although at significantly reduced levels. Surprisingly, sufficient MIS was produced to eliminate the Müllerian ducts. In contrast, males homozygous for the mutant SOX9-binding site did not initiate *Mis* transcription, resulting in pseudohermaphrodites. These studies suggest an essential role for SOX9 in the initiation of *Mis* transcription, whereas SF1 appears to act as a quantitative regulator of *Mis* transcript levels, perhaps for influencing non-Müllerian duct tissues. Comparative studies of *Mis* expression in vertebrates indicate that the *Mis* promoter receives transcriptional inputs that vary between species but result in the same functional readout.

Introduction

Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), is an essential component of the male sexual differentiation pathway in reptiles, birds, and mammals (Josso et al., 1993). In males, MIS causes the regression of the Müllerian ducts, which in mammals are the progenitors of the oviduct, uterus, and upper portion of the vagina. MIS is secreted by Sertoli cells of the fetal and prepubertal testis. In male mice, *Mis* transcripts are first detected after embryonic day 11.5 (E11.5), increasing through E19.5, then decreasing dramatically after birth (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995). In female mice, *Mis* transcripts are first detected 6 days after birth in the granulosa cells of the ovary. This strict temporal and quantitative regulation of *Mis* expression is essential for the elaboration of the male and female phenotypes.

The *Mis* gene has been cloned in diverse vertebrate species (Cate et al., 1986; Picard et al., 1986; Münsterberg and Lovell-Badge, 1991; Haqq et al., 1992; Carré-Eusèbe et al., 1996; Neeper et al., 1996; Wibbels et al., 1998; Western et al., 1999). Mutations in the *Mis* gene have been detected in human males with persistent Müllerian duct syndrome (PMDS), a rare form of pseudohermaphroditism, that is characterized by the retention of female reproductive organs in an otherwise virilized male (Josso et al., 1997). In mice, mutation of the *Mis* gene also causes males to develop as internal pseudohermaphrodites (Behringer et al., 1994). Although human males and their murine counterparts with PMDS can be sterile, their ontogenies are different. In humans, sterility is caused by testicular dystrophy as a result of cryptorchidism (Josso et al., 1997), whereas mice are infertile because of superimposed male and female reproductive organs blocking sperm delivery (Behringer et al., 1994).

Sequential deletion of the 3.5 kb region immediately upstream of the mouse *Mis* transcription start site identified a 180 bp fragment immediately adjacent to the start site that is capable of directing the expression of a reporter gene in primary rat Sertoli cells (Shen et al., 1994). Sequence comparisons of the 180 bp segment among different mammalian species identified a conserved 20 bp motif (MIS-RE-1) (Shen et al., 1994). This motif contains a nuclear hormone receptor half site (AGGTCA) that in vitro can bind the orphan nuclear hormone receptor steroidogenic factor 1 (SF1) (Lala et al., 1992; Honda et al., 1993) (Figure 1A). Electrophoretic mobility shift assays (EMSA) showed that mutation of the half site to ATTTCA abolished SF1 binding. In addition, cotransfection assays indicated that SF1 binds the half site and activates *Mis* transcription (Shen et al., 1994). The 180 bp fragment was also found to direct the expression of a reporter gene in an *Mis*-specific pattern in transgenic mice (Giuili et al., 1997). However, no reporter expression was detected when the half site within the 180 bp fragment was mutated. In mice, SF1 is expressed in the genital ridges of both sexes beginning at E9.0 (Ikeda et al., 1994). As sexual differentiation proceeds, SF1 is upregulated in the fetal testis and regulated to very low levels in the ovaries. Taken together, these studies argue that SF1 is essential for activating *Mis* transcription.

A conserved high mobility group (HMG) transcription factor–binding site is also present in the *Mis* promoter approximately 50 bp upstream from the SF1-binding site (Figure 1A). It has been suggested that the testis-determining factor SRY may regulate *Mis* transcription (Haqq et al., 1994), but other studies have failed to show any direct binding of SRY to the *Mis* promoter (Shen et al., 1994). An *Sry*-related HMG box gene, *Sox9*, is expressed in sites of chondrogenesis, the central nervous system, and other tissues, including the genital ridge (Wright et al., 1995; Kent et al., 1996; Morais da Silva et al., 1996). In mice, at E10.0, *Sox9* is expressed in the genital ridges of both male and female embryos. However, at E11.0, *Sox9* expression is upregulated in the male gonad and decreases to undetectable levels in female gonads (Kent et al., 1996; Morais da Silva et al., 1996). Humans with heterozygous null mutations for

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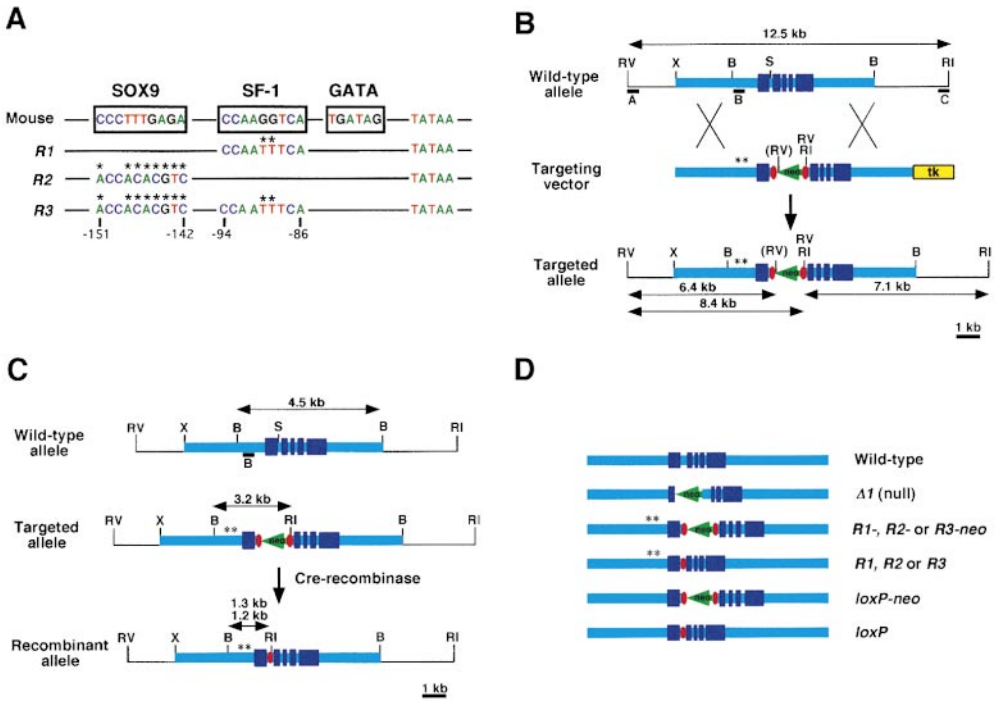

Figure 1. *Mis* Regulatory Mutagenesis

(A) Conserved transcription factor–binding sites in the mouse *Mis* promoter region. Mutations were introduced in the SF1-binding site (*R1*, *regulatory mutation-1*), the SOX9-binding site (*R2*, *regulatory mutation-2*), and in both SF1- and SOX9-binding sites in *cis* (*R3*, *regulatory mutation-3*).

(B) Strategy for generating targeted *Mis* regulatory mutations. The mouse *Mis* gene contains five exons, which are indicated by the dark blue boxes. The targeting vector possesses 7.3 kb of total homology (blue line). Either a loxPneo1 or PGKneobpA-lox *neo* expression cassette (*neo*) was introduced into the SfiI site of intron 1. The *neo* cassettes introduce unique EcoRI and EcoRV restriction sites for Southern blot analysis. A *tk* expression cassette was included for negative selection. An identical vector was also constructed but without the promoter mutations as a *loxP* control. External probes A and C were used for Southern analysis to screen for targeted ES cell clones. The sizes of the expected restriction fragments recognized by the probes are indicated. (RV), EcoRV site from *neo* cassette in *R1-neo* and *loxP-neo* targeting vectors. RI and RV, EcoRI and EcoRV sites from *neo* cassette in *R2-neo* and *R3-neo* targeting vectors.

(C) Removal of the *neo* cassettes. The *neo* cassettes were removed either in vivo by crosses with Cre-expressing transgenic mice or in vitro by transient expression of Cre recombinase. The recombination events were detected by Southern analysis of genomic DNA by digestion with EcoRI and BamHI and hybridized with probe B. **, mutations; B, BamHI; RI, EcoRI; RV, EcoRV; S, SfiI; X, XhoI.

(D) Summary of *Mis* alleles.

SOX9 develop campomelic dysplasia, a lethal bone malformation syndrome (Foster et al., 1994; Wagner et al., 1994). Interestingly, approximately 75% of XY *SOX9* heterozygotes also show sex reversal (Mansour et al., 1995). EMSA and cotransfection experiments have demonstrated that SOX9 can bind the conserved HMG site and activate *Mis* transcription in COS cells (De Santa Barbara et al., 1998; Morais da Silva and R. L.-B., unpublished observations). Furthermore, these studies showed that SOX9 and SF1 could act synergistically to activate the *Mis* promoter. Thus, *Mis* transcription may have multiple regulators to create the complex expression pattern found in male and female mammals.

Although a number of genes involved in sexual development have been molecularly defined, their precise positions and relationships in this developmental pathway remain unclear (Swain and Lovell-Badge, 1999). In vitro assays are useful for suggesting which factors can interact with specific *cis* elements to regulate gene transcription. However, their in vivo relevance cannot be determined using these assays. Although transgenic animals provide a useful in vivo assay system for investigating the activity of transcription factor–binding sites, the transgenes are of limited length and integrate at ectopic sites within the genome that often influence expression. To determine the true in vivo function of the conserved SF1- and HMG-binding sites within the *Mis* promoter, we have mutated these sites in the endogenous mouse *Mis* promoter by gene targeting in ES cells. Our findings define a complexity in the regulation of *Mis* not detected in in vitro and transgenic mouse experiments. Furthermore, our in vivo findings in the mouse, together with recent *Mis* expression studies in birds and reptiles, reveal that the SOX9- and SF1-mediated regulation of *Mis* transcription is divergent between mammalian and nonmammalian amniote vertebrates. This illustrates how rapidly evolving sex-determining mechanisms can still lead to the same downstream events critical for vertebrate sexual development.

Results

Generation of Mice with Targeted *Mis* Promoter Mutations

To generate mice with specific transcription factor–binding site mutations in the endogenous *Mis* promoter,

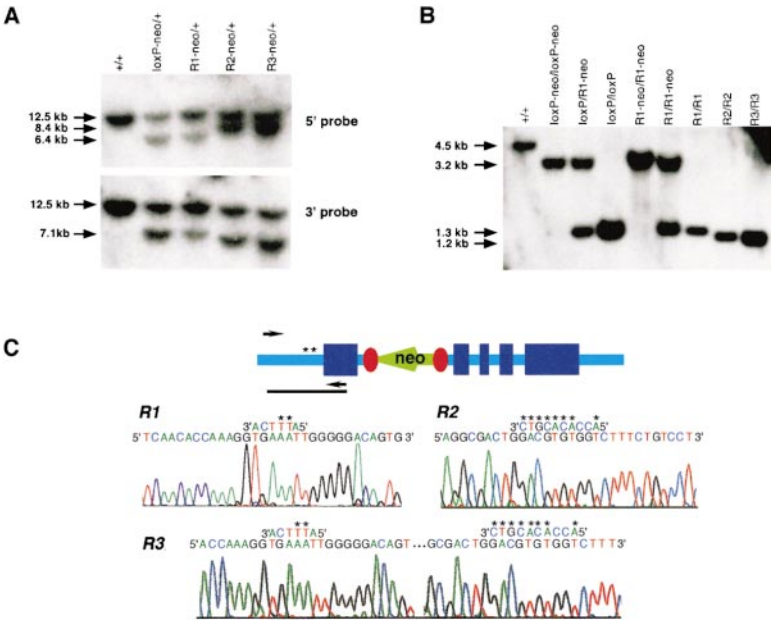

Figure 2. Screening of *Mis* Regulatory Mutant Alleles

(A) Southern blot analysis of *Mis* targeted clones. *R1-neo* or *loxP-neo* targeted clones digested with EcoRI/EcoRV produce 12.5 kb wild-type and 6.4 kb targeted bands when hybridized to an external *Mis* 5′ probe. *R2-neo* and *R3-neo* targeted clones digested with EcoRI/EcoRV produce 12.5 kb wild-type and 8.4 kb targeted bands when hybridized to the external *Mis* 5′ probe. All targeted clones digested with EcoRI/EcoRV produce 12.5 kb wild-type and 7.1 kb targeted bands when hybridized to an external *Mis* 3′ probe.

(B) Southern blot genotyping of tail DNA from males with *Mis* regulatory mutations. EcoRI/BamHI digested genomic DNA was hybridized with probe B. Shown are 4.5 kb wild-type, 3.2 kb *neo* positive alleles, and 1.3/1.2 *neo* negative alleles.

(C) Sequence analysis of the *Mis* promoter region. PCR primers (arrows) were designed to flank and amplify the mutated region. DNA sequencing was performed on the targeting vectors, targeted ES cell clones, and homozygous mutant animals (shown) to detect the presence of the mutations and the absence of any unwanted modifications introduced during the mutagenesis strategy.

we used a Cre recombinase/*loxP* gene targeting strategy. In this strategy, the transcription factor–binding site mutations were introduced by site-directed mutagenesis into the 5′ arm of homology of the targeting vector (Figures 1A and 1B). The alterations in the transcription factor–binding sites did not include base pair additions or deletions because the exact spacing between the conserved elements may be functionally important. For positive selection, a *loxP*-flanked (floxed) *neo* expression cassette was placed within intron 1 in reverse orientation relative to *Mis* transcription. The presence of exogenous selectable marker sequences can influence the activity of a targeted locus (Pham et al., 1996). Therefore, after targeted ES cell clones or mice containing the *Mis* alleles with the *neo* cassette were obtained, the *neo* cassette was removed by Cre recombinase expression, leaving a single *loxP* site within intron 1 (Figure 1C). Mice that had a wild-type *Mis* promoter region and a single *loxP* site within intron 1 were also generated to control for the potential effects that a single *loxP* site in intron 1 might have on *Mis* transcription.

Mutant and control gene targeting vectors differing in their SF1 and SOX9 response elements were generated. The SF1-binding site mutation we generated in the *Mis* promoter was identical to the 2 bp mutation previously documented in in vitro and transgenic mouse studies (Shen et al., 1994; Giuili et al., 1997) (Figure 1A). The mutant SOX9-binding site was created by scrambling the sequence (Figure 1A). This prevented SOX9 binding as shown in gel shift assays (Ryohei Sekido and R. L.-B., data not shown). The mutant targeting vectors and their resulting *Mis* alleles were designated *regulatory mutation-1-neo* (*R1-neo*) for the SF1-binding site mutation with the *neo* cassette, *regulatory mutation-2-neo* (*R2-neo*) for the SOX9-binding site mutation with the *neo* cassette, and *regulatory mutation-3-neo* (*R3-neo*) for the in *cis* SF1- and SOX9-binding site mutations with the *neo* cassette. The *Mis* regulatory mutations in which the *neo* cassette has been removed by Cre recombinase were designated *R1*, *R2*, and *R3*, respectively. The control *Mis* allele with the *neo* cassette was designated *loxP-neo*. The *Mis* control allele in which the *neo* cassette has been removed was designated *loxP*. A summary of the *Mis* alleles generated in this study are listed in Figure 1D.

Targeted ES cell clones were initially identified by Southern analysis (Figure 2A). Targeted ES cell clones were then analyzed by PCR and DNA sequencing to confirm the presence of the SF1- and SOX9-binding site mutations and to ensure that no other changes were introduced into the promoter region (Figure 2C). Correctly targeted ES cell clones were microinjected into blastocysts. A total of ten independent ES cell clones representing at least two each of the mutant and control vectors contributed to the germline of the resulting chimeras.

The SF1-Binding Site within the *Mis* Promoter Is Not Essential for Müllerian Duct Regression

The reproductive tracts of 6-week-old males carrying either the SF1-binding site mutation *R1-neo* or the control *loxP-neo* alleles were analyzed (Figure 3). Male mice heterozygous for *R1-neo* or *loxP-neo* had normal reproductive tracts. In contrast, both *R1-neo*/*R1-neo* and *loxP-neo*/*loxP-neo* males developed as internal pseudohermaphrodites retaining Müllerian duct derivatives (a uterus and oviducts) in addition to their normal male reproductive organs (Figure 3). This phenotype was identical to the reproductive tract abnormalities previously reported for *Mis*-null male mice (Behringer et al., 1994). These results suggest that the *neo* cassette was disrupting *Mis* expression.

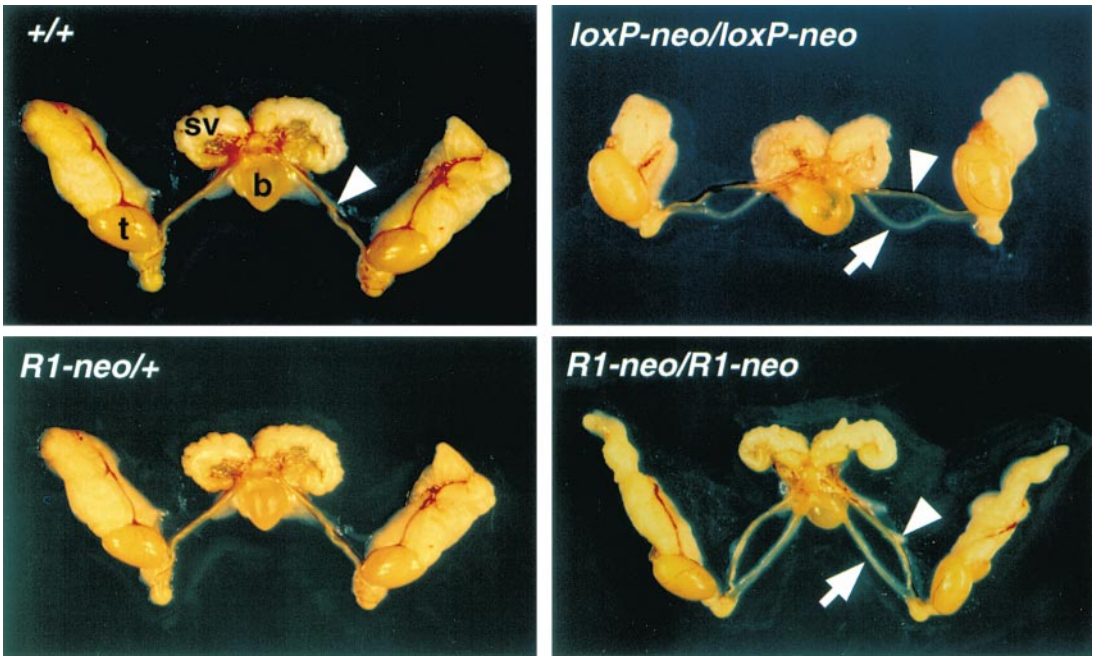

Figure 3. Gross Dissection of the Internal Reproductive Organs of Adult Male Mice with *MIS-neo* Alleles Reveals Pseudohermaphroditism

Wild-type (+/+) and *R1-neo*/+ mice show a normal phenotype. The *loxP-neo/loxP-neo* and *R1-neo/R1-neo* mice develop as pseudohermaphrodites with a uterus (arrow) and oviducts. The male and female reproductive organs run parallel to each other joined by connective tissue that has been cut to reveal the dual nature of the organs. Arrowhead, vas deferens; b, bladder; sv, seminal vesicle; t, testes.

To eliminate interference by the *neo* cassette on *Mis* transcription, the floxed *neo* cassette was removed from the *R1-neo* and *loxP-neo* alleles (Figure 2B). The reproductive tracts of 6-week-old *R1*/*R1* and *loxP*/*loxP* males were dissected and analyzed (Figure 4). Gross examination of *R1*/*R1* and *loxP*/*loxP* males revealed the presence of only male reproductive organs (Figure 4A). Closer observation showed that male mice homozygous for either *R1* or *loxP* alleles have normal reproductive tracts with no retention of Müllerian duct–derived tissues (Figure 4B). These results indicate that the SF1-binding site in the *Mis* promoter is dispensable for normal male sexual development. We next combined the *R1-neo* allele (functionally equivalent to a null) with the *loxP* and *R1* alleles to further reduce *Mis* expression. *loxP/R1-neo* compound heterozygous males were normal with complete Müllerian duct regression (Figure 4B). Interestingly, the Müllerian ducts of *R1/R1-neo* compound heterozygous males were only partially regressed (Figure 4B). Although the *R1/R1-neo* males partially retained uterine tissue, they all proved to be fertile.

To document that the residual tissue in the *R1/R1-neo* males was truly uterine tissue of Müllerian duct origin, a *lacZ* reporter cassette was introduced into the *Mis type II receptor* gene by homologous recombination. The targeted allele was designated MIS receptor knockin (*MRKI*). *MRKI*/+ females express β-galactosidase (β-gal) activity during embryogenesis in the Müllerian ducts and subsequently in the adult in the uterus and oviducts (Figure 4C). The details of these mice will be reported elsewhere. *R1/R1* males were mated to *R1-neo*/+ females carrying *MRKI* to generate *R1/R1-neo*; *MRKI*/+ males. After X-gal staining, the retained presumptive uterine tissue in 6-week-old *R1/R1-neo*; *MRKI*/+ males stained positive for β-gal activity, while these structures in *R1/R1-neo* males without the *MRKI* marker were negative for β-gal activity (Figure 4C). The reproductive tracts of the *R1/R1-neo*; *MRKI*/+ males were embedded in paraffin and sectioned. In +/+; *MRKI*/+ adult females, β-gal activity was detected in the longitudinal muscle layer of the uterine body and horns. The retained presumptive uterine tissue in the *R1/R1-neo*; *MRKI*/+ males expressed β-gal activity in an identical pattern (data not shown). Taken together, these results indicate that the retained tissue in the *R1/R1-neo* males is of Müllerian duct origin and is composed of the uterine body and proximal uterine horns. These genetic results indicate that the *R1* allele is a hypomorph with reduced *Mis* expression relative to wild type.

The SF1-Binding Site within the *Mis* Promoter Is Required for the Upregulation of *Mis* Transcription

MIS is secreted by the fetal testis and may act first on the region of the Müllerian duct closest to the testis, requiring either a constant production or higher concentrations, or both, of MIS to reach the farthest region of its target organ to cause it to regress. Müllerian ducts are only sensitive to MIS action during a narrow window in development, suggesting that either low or delayed *Mis* expression from the *R1* allele may be responsible for the partial regression of the Müllerian ducts in the *R1/R1-neo* males.

To compare the onset of *Mis* expression from the *R1* allele to that of the wild-type allele, we performed RT–PCR and RNase protection assays (RPA) on embryonic and postnatal testes. RNA was extracted from E11.5 and E12.5 *R1/R1* and +/+ genital ridges. Because

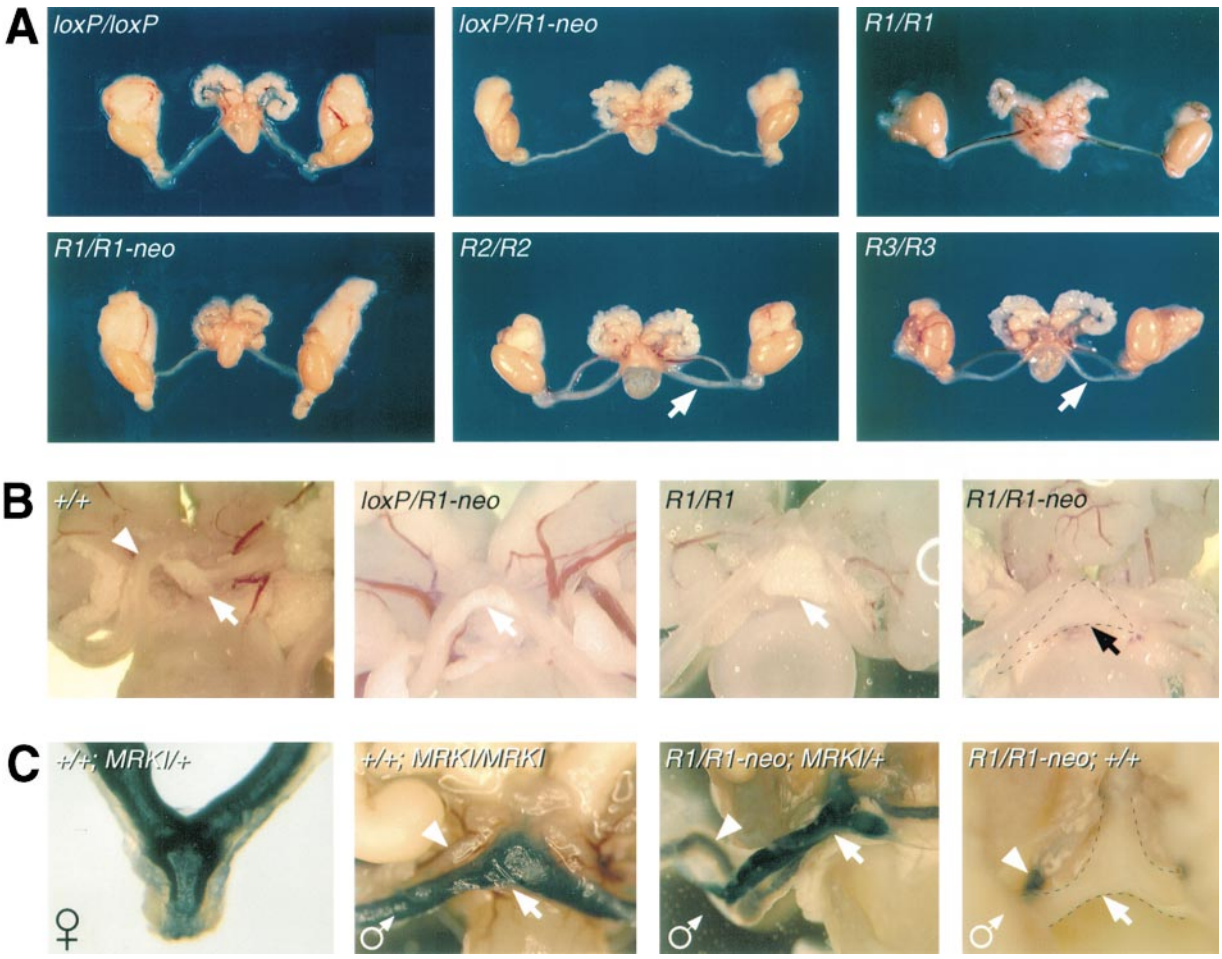

Figure 4. Phenotype of Adult Male Reproductive Tracts with Control or Mutant *Mis* Alleles after *neo* Removal

(A) *loxP/loxP* (control) and *R1/R1* males as well as the compound heterozygous *loxP/R1-neo* (control) and *R1/R1-neo* males are grossly normal. *R2/R2* and *R3/R3* animals develop as pseudohermaphrodites, characterized by the retention of female reproductive organs in an otherwise virilized male. The arrow points to the uterus.

(B) Partial retention of Müllerian duct tissue in *R1/R1-neo* males. Detailed view of the male reproductive organs. *R1/R1* and control *loxP/R1-neo* males have a wild-type (+/+) phenotype (arrow, adipose tissue; arrowhead, vas deferens). *R1/R1-neo* males have apparently partially retained uterine structures (arrow).

(C) Persistent uterine tissue in *R1/R1-neo* males. To provide conclusive evidence on the origin of the retained tissue, a *lacZ* knockin into the *MIS type II receptor gene* (*MRKI*) was utilized. *MRKI* is expressed as β-gal activity in the *MRKI/+* female uterus. *MRKI/MRKI* males are pseudohermaphrodites because the MIS type II receptor is essential for Müllerian duct regression (Imbeaud et al., 1995; Mishina et al., 1996). The retained uterus in *MRKI/MRKI* males expresses β-gal activity (arrow). The retained tissue of the *R1/R1-neo*; *MRKI/+* males also expresses β-gal activity (arrow), demonstrating that the tissue is of Müllerian duct origin. As a control, *R1/R1-neo* males without *MRKI* were also analyzed. The retained tissue (arrow) does not have endogenous β-gal activity. Arrowhead, vas deferens.

sexing by morphology is not possible until E12.5, PCR amplification of the Y chromosome–specific gene *Sry* was performed to determine the sex of the E11.5 embryos (Gubbay et al., 1990). *Mis* expression was first detected at E12.5 in both *R1/R1* and +/+ testes by RT–PCR (Figure 5A). These results indicate that the onset of *Mis* expression is not affected in the *R1/R1* mutants. We next quantitated *Mis* expression in *R1/R1* testes during embryogenesis and postnatal development by RPA (Figure 6A). *R1/R1*, *R1/R1-neo*, and +/+ RNA was isolated from E13.5, E15.5, 9 dpp, and 12 dpp testes. As a control *loxP/loxP* RNA from E15.5 and 12 dpp testes was also isolated. RPAs showed that the pattern of expression from the wild-type *Mis* allele was similar to that previously reported (Hacker et al., 1995). In contrast, there was a dramatic reduction in *Mis* transcript levels from the *R1* allele. Interestingly, the same low level of expression was maintained until 12 dpp when it became undetectable, a stage when wild-type and *loxP* alleles were still being transcribed. Quantitation of the *Mis* transcript levels showed a greater than 3-fold decrease in the levels of *Mis* expression from the *R1* allele in comparison with controls (Figure 6B). These results provide a molecular confirmation that expression from the *R1* allele is reduced.

The SOX9-Binding Site within the *Mis* Promoter Is Essential for Müllerian Duct Regression

We next explored the role of the conserved SOX9-binding site in the *Mis* promoter on the regulation of *Mis*

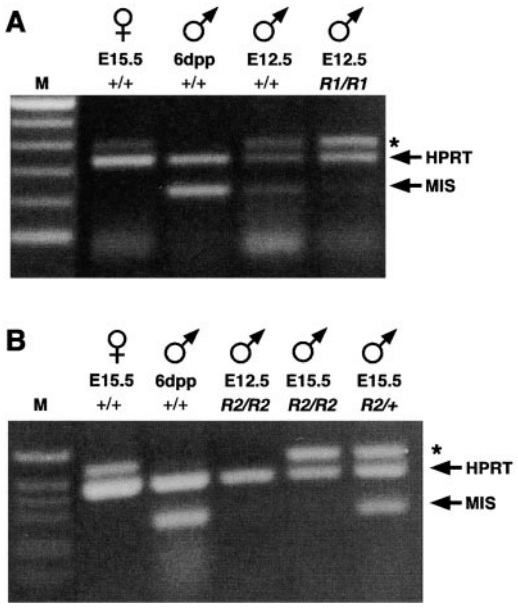

Figure 5. RT–PCR Analysis of *Mis* Transcription in the Fetal Testes of *R1/R1* and *R2/R2* Mice

(A) RT–PCR shows a normal onset of *Mis* transcription in *R1/R1* testes. Embryonic ovaries and postnatal testes served as negative and positive *Mis* controls, respectively. Ubiquitously expressed *Hprt* was used as an internal control. The upper band (*) in some lanes is amplified genomic DNA for the *Mis* locus.

(B) RT–PCR shows the absence of *Mis* transcripts in *R2/R2* testes, whereas *R2/+* testes express *Mis*.

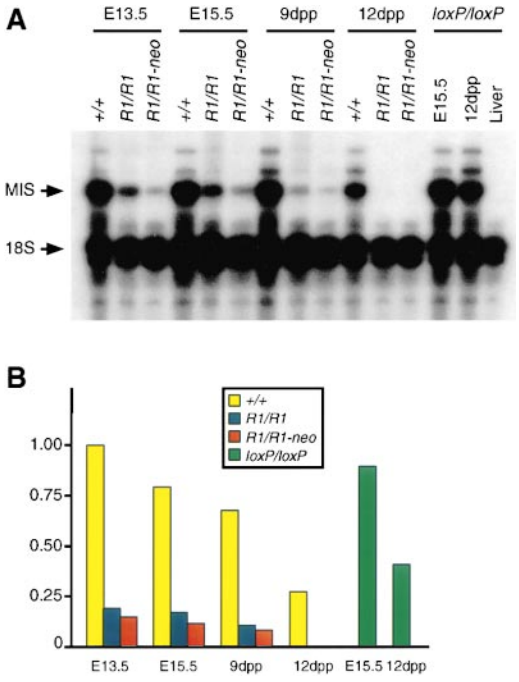

Figure 6. *Mis* Expression Levels from the *R1* Allele

(A) RPA analysis of *Mis* transcription from the *R1* and *loxP* alleles in testes. RPA shows that the level of transcription from the *R1* allele in testes is significantly lower than that of wild type during embryogenesis and postnatal development. At 12 dpp, a time when wild-type *Mis* is still being expressed, no *Mis* transcripts are detected from the *R1* allele. *loxP/loxP* testicular RNA was used to measure any effect that the *loxP* sequence might have on *Mis* transcription. Liver RNA, negative control; ribosomal 18S probe, internal control.

(B) Quantitation of *Mis* RNA levels. Although *Mis* levels in *R1/R1* testes (blue bar) are more than 3-fold lower than wild type (yellow bar), these animals have completely regressed Müllerian ducts. *R1/R1-neo* males (red bar) have an additional slight decrease in *Mis* levels, and, as shown, they partially retain Müllerian duct tissue. *loxP/loxP* controls (green bar) express wild-type levels of *Mis* transcripts.

transcription. Males homozygous for the *R2* allele were sacrificed at 6 weeks of age, and their reproductive tracts were dissected and analyzed. *R2/R2* and *R2/R1-neo* males were internal pseudohermaphrodites with complete retention of Müllerian duct–derived organs (Figure 4A), identical in phenotype to *Mis*-null male mice. These results suggest that the expression of *Mis* from the *R2* allele is below the threshold required for complete Müllerian duct regression. Perhaps not surprisingly, the *R3* allele, which includes both SF1- and SOX9-binding site mutations in *cis*, behaved exactly like the *R2* allele.

The SOX9-Binding Site within the *Mis* Promoter Is Essential for Transcriptional Initiation

We next examined the molecular phenotype of the *R2* allele. To determine the onset of *R2* expression, we collected RNA from E12.5 testes, a time when *Mis* expression from the wild-type and *R1* alleles was first detected. RT–PCR showed no detectable transcripts from *R2/R2* testes, indicating an absence of *Mis* transcription initiation at E12.5 (Figure 5B). To determine whether the onset of *Mis* transcription from the *R2* allele had been delayed to a later embryonic time point, we collected E15.5 testicular RNA. E15.5 was chosen because this is the time when Müllerian ducts are actively regressing. RT–PCR showed an absence of *Mis* transcripts in *R2/R2* testes, whereas expression was observed in *R2/+* testes (Figure 5B). These results demonstrate that the SOX9-binding site is essential for *Mis* transcription in vivo.

Discussion

In mammals, *SRY* initiates the conversion of the undifferentiated fetal gonad into a testis (Goodfellow and Lovell-Badge, 1993). However, in birds and reptiles, no *SRY* gene has been identified, suggesting that other mechanisms initiate the formation of testes in these vertebrates. Yet in both mammalian and nonmammalian amniotes, MIS and testosterone are subsequently secreted by the developing testis to mediate the regression of the Müllerian duct system and virilization of the internal and external genitalia, respectively. Thus, the MIS signaling pathway for Müllerian duct regression is one of the primary and essential processes of male sexual development.

The *Mis* gene is expressed in a complex, sexually dimorphic pattern. This complex expression pattern is likely to be regulated by numerous transcriptional inputs. We have analyzed the in vivo relevance of two evolutionarily conserved transcription factor–binding sites within the mouse *Mis* promoter. These regulatory

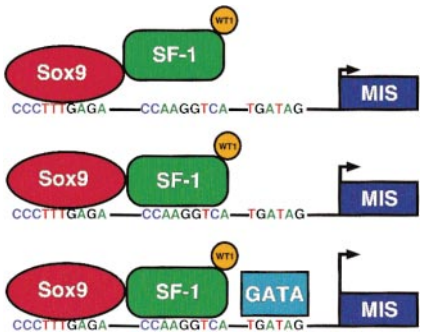

Figure 7. Model for *Mis* Transcription in Sertoli Cells of the Male Gonad

The current data suggest that *Mis* transcription is initiated by SOX9 acting through the conserved HMG site of the *Mis* promoter. In the absence of an HMG-binding site, no *Mis* transcription is initiated, leading to the retention of Müllerian duct–derived tissues. After the initiation of *Mis* transcription by SOX9, expression is upregulated by SF1 binding to the nuclear hormone half site and physical interaction with SOX9. The upregulation of *Mis* transcription by SF1 may function to regulate non-Müllerian duct cell types, such as Leydig cells. A specific isoform of WT1 has been shown to physically interact with SF1 to synergistically upregulate *Mis* transcription. In the absence of an SF1-binding site, SF1 is unable to bind the *Mis* promoter, resulting in the observed decrease in *Mis* levels. These levels are still sufficient for complete regression of the Müllerian ducts. GATA4 may also have a role in the initiation of *Mis* transcription through binding of the conserved GATA site, perhaps through interactions with SOX9. However, the timing of GATA4 expression indicates that it, like SF1, may also function as a quantitative regulator of *Mis* transcription. GATA4 levels upregulate in males at E13.5 and downregulate after birth, correlating with the postnatal decrease in *Mis* transcription. GATA1 levels initiate soon after birth and correlate with the decrease in GATA4 and *Mis* levels. Thus, the conserved GATA site in the *Mis* promoter may interact with both GATA1 and GATA4 to modulate *Mis* levels.

mutants have revealed complexities not detected in vitro, nor even in standard transgenic mouse assays. Our in vivo results indicate a hierarchy of transcription factors controlling the expression of *Mis* in Sertoli cells that is essential for proper development of the male phenotype. A model for *Mis* transcription based upon our findings and previously published results is presented in Figure 7.

SF1 Is a Quantitative Regulator of *Mis* Transcription but Is Not Essential for Müllerian Duct Regression

Our in vivo results obtained in the context of the natural locus demonstrate that the SF1-binding site in the *Mis* promoter is not required for the initiation of *Mis* transcription, but rather for its upregulation during embryogenesis. Surprisingly, although males homozygous for the SF1-binding site mutation (*R1/R1*) had dramatically reduced levels of *Mis* transcripts, regression of the Müllerian duct system proceeded to completion. It was not until the *R1* and null alleles were combined, dropping the levels of *Mis* even further past a critical threshold, that Müllerian duct tissue was partially retained. Interestingly, the Müllerian duct–derived tissues that persisted were those farthest away from the source of MIS production, the testes. These results are consistent with the idea that MIS may function in a local manner for Müllerian duct regression rather than as a circulating hormone. Similar findings were reported by Jost (1953) who showed that testicular grafts into female fetal rabbits caused the regression of the Müllerian ducts only on the grafted side of the fetus. However, the regression of the Müllerian duct system in the bovine freemartin suggests that MIS can also act as a circulating hormone. More studies will be required to determine how MIS reaches its target organs. The *R1/null* male mice were fertile despite the low *Mis* levels and the presence of some Müllerian duct–derived structures. Male North American beavers typically retain residual uterine tissue (Meier et al., 1998). This raises the possibility that there may be fertile human males with variable levels of MIS that unknowingly retain some uterine tissue.

The observation that male mice homozygous for the SF1-binding site mutation have complete regression of the Müllerian duct system despite significantly reduced levels of *Mis* expression demonstrates that normal MIS levels are in many fold excess. Perhaps this excess serves as a guarantee that males do not differentiate female reproductive organs that would compromise fertility. Another possibility, which is not mutually exclusive, is that SF1 upregulates *Mis* expression to levels necessary for its action on non-Müllerian duct tissues.

The MIS type II receptor is expressed in the mesenchymal cells surrounding the Müllerian ducts, Sertoli and Leydig cells of the testis, and granulosa cells of the ovary (Baarends et al., 1994, di Clemente et al., 1994; Racine et al., 1998; Lee et al., 1999). High levels of MIS have been shown in transgenic mice to reduce circulating testosterone levels, sometimes causing an absence of virilization (Behringer et al., 1990; Lyet et al., 1995). The high levels of MIS in these transgenic mice caused a disruption in the differentiation of Leydig cell precursors (Racine et al., 1998). In addition, a proportion of older *Mis*-null mice develop Leydig cell hyperplasia (Behringer et al., 1994). Furthermore, an MIS deficiency leads to enhanced testicular tumorigenesis, including Leydig cell tumors, when combined with an α-inhibin deficiency (Matzuk et al., 1995). Thus, the upregulation of *Mis* caused by SF1 in the male fetus may serve to modulate Leydig cell function and not Müllerian duct regression.

Males homozygous for the SF1-binding site mutation initiated *Mis* transcription with correct timing that was maintained throughout embryogenesis, suggesting that SF1 is not essential for the maintenance of *Mis* transcription. However, *Mis* transcript levels from the *R1* allele became undetectable by 12 dpp, a time when wild-type *Mis* transcripts are still detected (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995). Thus, it is possible that SF1 may be required for the postnatal maintenance of *Mis* transcription. Alternatively, because *Mis* transcription from the *R1* allele is generally low, the lack of *Mis* transcripts at 12 dpp may be due to the normal downregulation of *Mis* after birth (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995).

One factor that may interact with SF1 to upregulate *Mis* transcription is the Wilms' tumor suppressor, WT1 (Call et al., 1990). Both SF1 and WT1 are required in mice for the formation of gonads (Kreidberg et al., 1993; Luo et al., 1994). Interestingly, a specific isoform of WT1 has been reported to physically interact with SF1 to synergistically activate the *Mis* promoter through the SF1-binding site in vitro (Nachtigal et al., 1998). The

synergism between SF1 and WT1 for *Mis* transcription can be antagonized by the nuclear receptor DAX1 through physical interactions with SF1 (Muscatelli et al., 1994; Swain et al., 1996; Nachtigal et al., 1998). Thus, SF1 may serve as a nexus for modulating *Mis* expression levels once transcription has been initiated.

It is formally possible that *Mis* transcription in the testes of *R1/R1* males could also be explained by the existence of redundant SF1-binding sites. However, in vitro studies have demonstrated the absence of additional functional SF1-binding sites within −3.2 kb from the *Mis* transcription start site (Giuili et al., 1997). We have scanned the *Mis* gene for other putative SF1-binding sites and have found related sites. However, these sites differ from the consensus SF1 binding sequence either within or at the first three nucleotides 5′ of the half site. These three nucleotides are necessary for SF1 binding specificity (Morohashi et al., 1992; Wilson et al., 1993). Interestingly, the *Sap62* locus is approximately 300 bp immediately upstream of the *Mis* transcription start site (Dresser et al., 1995). *Sap62* encodes a spliceosome protein that is ubiquitously expressed and is transcribed in the same orientation as *Mis*. Thus, a ubiquitously expressed gene resides adjacent to a gene that is expressed in a complex pattern. The coevolution of these two genes may have imposed physical constraints on the placement of the *Mis* regulatory elements, forcing them to be located within this small intergenic region. Regardless of the presence of other functional SF1-binding sites associated with the *Mis* locus, we show that the site located within the proximal promoter is required for increasing *Mis* transcript levels.

It is still possible that SF1 may contribute to the low levels of *Mis* expression observed in the *R1/R1* testes through its physical interaction with SOX9 (De Santa Barbara et al., 1998). Thus, it may form an essential part of a transcription factor complex at the *Mis* promoter, without direct contact with DNA, as suggested for WT1 (Nachtigal et al., 1998). This issue may be resolved by generating a Sertoli cell–specific inactivation of *Sf1* gene function.

SOX9 Is Required to Initiate *Mis* Transcription

Haqq et al. (1994) showed that SRY could activate a 114 bp human *Mis* promoter in vitro. Although mutation of the SRY protected region abolished SRY binding, it did not alter the transcriptional response of the *Mis* promoter to SRY, suggesting that SRY does not directly regulate *Mis* transcription. In contrast, in vitro studies have demonstrated the necessity of SOX9 and its binding site in the *Mis* promoter for transcriptional activity (De Santa Barbara et al., 1998). However, recent in vitro studies have questioned the role of SOX9 in the sex-specific regulation of *Mis* (Tremblay and Viger, 1999).

Our in vivo results demonstrate that the SOX9-binding site within the mouse *Mis* promoter is essential for the initiation of *Mis* transcription. Male mice homozygous for the SOX9-binding site mutation completely retained Müllerian duct–derived organs due to a complete absence of *Mis* expression. This suggests that SOX9 is an essential activator of *Mis* transcription. Thus, mutations in the SOX9-binding site of the human *Mis* promoter may provide a molecular explanation for MIS-deficient individuals with PMDS that lack mutations within the *Mis* coding region (Josso et al., 1997). Evidence from human genetics suggests that *SOX9* is a key sex-determining gene (Foster et al., 1994; Wagner et al., 1994). Our results suggest that in addition to its sex-determining role, SOX9 also regulates *Mis*, a gene product that is directly involved in sexual differentiation. Thus, in mammals, SOX9 may be a master regulator of sexual development.

There must be other factors, in addition to SOX9, that are required to activate *Mis* transcription specifically in fetal Sertoli cells because SOX9 is expressed in many other tissues that do not express *Mis* (Wright et al., 1995; Kent et al., 1996; Morais da Silva et al., 1996). The SOX9-containing transcription factor complex that initiates *Mis* transcription in fetal Sertoli cells may then be modified by other factors such as SF1 to modulate *Mis* transcription up or down. One transcription factor that may also cooperate with SOX9 to initiate or modulate *Mis* transcription is GATA4 (Arceci et al., 1993; Tremblay and Viger, 1999). A conserved GATA factor–binding site is located about 10 bp downstream from the SF1-binding site in the mammalian *Mis* promoters (Figure 1A). GATA4 can bind this site and activate the −180 bp *Mis* promoter in vitro (Viger et al., 1998). GATA4 protein is detected at E11.5 in the genital ridge of both male and female mice. In males, levels of GATA4 increase at E13.5, are maintained during embryogenesis, but are then downregulated by 14 dpp (Viger et al., 1998; Ketola et al., 1999). In females, GATA4 expression is downregulated by E16.5 but is expressed in adult granulosa cells. Thus, it is possible that GATA4 may act with SOX9 to initiate *Mis* transcription. In vivo studies to address the role of the GATA-binding site on the transcriptional regulation of *Mis* are currently under investigation.

Defining Genetic Pathways of Vertebrate Sexual Development

Although in vitro and even transgenic mouse assays have indicated which factors potentially regulate *Mis* transcription, it has not been clear how these factors truly act in vivo. Our in vivo studies clearly define the requirement of the SOX9-binding site within the mouse *Mis* promoter for initiating *Mis* transcription and a quantitative role for the SF1-binding site. The high degree of sequence conservation within the promoter regions of the cloned mammalian *Mis* genes suggests that the activities of the transcription factor–binding sites that we have defined here are likely to be functionally conserved in mammals. Surprisingly, recent studies in the chick and alligator demonstrate that *Mis* transcripts are expressed prior to *SOX9* expression (Oreal et al., 1998; Western et al., 1999). Thus, in contrast to mammals, the initiation of *Mis* transcription appears to be independent of SOX9 in nonmammalian amniotes.

In eutherian mammals, sex determination is genetically regulated, whereas sexual differentiation is controlled by gonadal hormones. Thus, alterations in gonadal hormone levels in the fetuses of eutherian mammals have no effects on sex determination. In contrast, sex determination in amphibians, fish, reptiles, and birds can be greatly influenced by steroid hormones. For example, the administration to chick embryos of an aromatase

inhibitor that blocks the conversion of testosterone to estrogen can convert genetic females into fertile males (Elbrecht and Smith, 1992). In turtles, alligators, and lizards with temperature-dependent sex determination, estrogen treatment at male-producing temperatures can result in ovarian development (Pieau et al., 1994), whereas in turtles androgen treatment at female-producing temperatures can induce testis formation (Wibbels et al., 1992). In fish, androgen treatment can convert genetic females into fertile males, and estrogen treatment can convert genetic males into females (Baroiller et al., 1999). Thus, there are fundamental differences in sex determination pathways between mammalian and nonmammalian vertebrates. However, downstream events such as *Mis* expression and regression of the Müllerian duct system are conserved among amniote species. Interestingly, the sequence and organization of the chick *Mis* promoter region is dramatically divergent from the sequence of mammalian *Mis* promoters (Oreal et al., 1998). An SF1-binding site is located 5 bp upstream of the TATA box, putative HMG-binding sites are scattered more 5′ prime, and no obvious GATA-binding sites are apparent. It is intriguing to note that SF1 also regulates *cytochrome P450 steroid hydroxylase* genes that are involved in steroid hormone biosynthesis (Parker and Schimmer, 1997). In addition, the expression pattern of *Sf1* in the chick suggests an important role for SF1 in the initiation of *Mis* transcription but not for its upregulation (Smith et al., 1999). Taken together, these findings indicate that the *Mis* promoter receives varying transcriptional inputs depending upon the species that results in the same functional readout, Müllerian duct regression. Because MIS has such an important and unique role in vertebrate sexual development, the *Mis* promoter must evolve with changing circumstances. If the sex determination mechanism evolves, then *Mis* regulation must follow.

Experimental Procedures

Generation of Gene Targeting Vectors

Base pair substitutions in the nuclear hormone receptor half site and HMG-binding site in the *Mis* promoter were generated by site-directed mutagenesis by overlap extension using PCR (Ho et al., 1989). Wild-type control (*loxP-neo*) and mutant (*R1-neo*, *R2-neo*, and *R3-neo*) gene targeting vectors differing in their *cis*-regulatory elements were constructed. The vectors were sequenced to confirm the presence of the mutations and to ensure that no other changes were introduced. A floxed *neo* expression cassette (loxPneo-1) was inserted, in reverse orientation relative to the direction of *Mis* transcription, into an SfiI site in the first intron of the *loxP-neo* and *R1-neo* targeting vectors (Figure 1B). Another floxed *neo* expression cassette (PGKneobpA-lox) was inserted at the identical site in intron 1 for the *R2-neo* and *R3-neo* targeting vectors. The exogenous sequences that remain, once the PGKneobpA-lox cassette is deleted by Cre recombinase, are less than the residual sequence of the loxPneo-1 cassette. The region of vector homology begins at the XhoI site 5′ of the *Mis* gene and extends 3′ to a BamHI site, providing 7.3 kb of total homology. The 5′ arm of vector homology contains 4.0 kb from an XhoI site to the SfiI site in intron 1 and includes the promoter mutations. The 3′ arm contains a homology of 3.3 kb from the SfiI site on intron 1 to a 3′ BamHI site. A herpes simplex virus-*thymidine kinase* expression cassette (pGLN-2) was placed outside the 3′ arm of homology to enrich for homologous recombinants by negative selection (Mansour et al., 1988). Insertion of the *neo* cassettes brought in two unique restriction sites, EcoRI and EcoRV, that were exploited during Southern blot analysis.

Generation of Targeted ES Cell Clones and Germline Alleles

The *Mis* vectors were linearized with NotI outside the 3′ homology. AB1 ES cells (1.0×10^7) (McMahon and Bradley, 1990) were electroporated independently with 25 μg of each vector and selected for 10 days in G418 and FIAU medium. A total of 300–600 $G418^r$, $FIAU^r$ ES cell colonies for each vector were picked and analyzed by Southern blot (Ramirez-Solis et al., 1992) with 5′ and 3′ external probes as previously described (Behringer et al., 1994) (Figure 1C). For *loxP-neo* and *R1-neo*, correctly targeted ES cell clones were identified by the presence of additional 6.4 kb and 7.1 kb bands when digested with EcoRI/EcoRV and hybridized with 5′ and 3′ external probes, respectively (Figure 2A). ES cell clones targeted with either *R2-neo* or *R3-neo* were identified by the presence of additional 8.4 kb and 7.1 kb bands when digested with EcoRI/EcoRV and hybridized with the same 5′ and 3′ probes, respectively (Figure 2A). Targeting frequencies of 1/9, 1/9, 1/14, and 1/6 $G418^r$, $FIAU^r$ ES cell colonies screened for *R1-neo*, *R2-neo*, *R3-neo*, and *loxP-neo* were obtained, respectively.

Correctly targeted ES cell clones were injected into 3.5 days postcoitum (dpc) C57BL/6 (B6) blastocysts, which were in turn transferred into 2.5 dpc Swiss foster mothers. High-percentage chimeras (80%–100% agouti pigmentation) were obtained for all of the *Mis* clones. Male chimeras were bred to B6 females, and their agouti pups were genotyped to confirm germline transmission of the targeted alleles.

Cre Recombinase–Mediated Excision of *neo*

The *neo* expression cassettes were removed from the targeted alleles either by mating male chimeras, or their germline progeny, to B6 CMV-cre transgenic mice. The *Cre recombinase* transgene was subsequently segregated away from the *Mis* mutations by crossing to B6. In addition, *neo* was removed from an independent *R1-neo* allele in ES cells by transient expression of Cre recombinase. Correctly targeted *R1-neo* clones were electroporated with 25 μg of supercoiled pOG231 plasmid. Cells were grown for 10 days without selection. Two hundred colonies were picked and screened by Southern blot. One ES cell clone carrying the *R1* allele in which *neo* was removed in vitro contributed to the germline of chimeras. *R1*, *R2*, *R3*, and *loxP* animals were independently bred to homozygosity and crossed to animals carrying the *R1-neo* allele. A final sequencing screen for the mutations was performed on homozygous animals.

RNA Analysis

Total RNA from embryonic and postpartum tissue samples was isolated using either Qiagen RNeasy mini or midi kits, or as described by Chomczynski and Sacchi (1987). RT–PCR was performed using *Mis* primers to exon 2 (5′-GAGCTCTTGCTGAAGTTC-3′) and exon 3 (5′-CTGCTTGGTTGAAGGGTTAAG-3′) (Münsterberg and Lovell-Badge, 1991). *Hprt* primers (5′-CCTGCTGGATTACATTAA AGCACTG-3′) and (5-GTCAAGGGCATATCCAACAACAAAC-3′) were used as an internal control (Koopman, 1993). RNase protection assays (RPA) were performed using a 315 bp PstI-SacI *Mis* probe that overlaps the *Mis* transcriptional start site (Hacker et al., 1995). The plasmid containing the probe was linearized with HindIII and transcribed using an Ambion MAXIscript T7 kit. The riboprobe was purified on a 5% polyacrylamide 8 M urea gel, excised, and eluted from the gel with Ambion elution buffer. Between 2–3 μg of total RNA was hybridized for 12–18 hr at 60°C with 4×10^5 cpm of RNA probe according to the manufacturer's instructions. Samples were electrophoresed on 5% polyacrylamide 8 M urea gels. Gels were autoradiographed overnight and then exposed to PhosphoImager screens (Molecular Dynamics) for approximately 8 hr for quantitative analysis.

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